

PRELIMINARY STUDIES ON THE USE OF MONOCLONAL ANTIBODIES AS PROBES FOR SYMPATHETIC DEVELOPMENT

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SUMMARY

The precise structural organization and proper functioning of the adult nervous system depend on the ability of neurones to make highly ordered synaptic connexions. To define molecules involved in the development of these connexions and to study their functional roles, we use primary cultures of dissociated rat sympathetic neurones grown in the virtual absence of non-neuronal cells. These neurones can develop adrenergic or cholinergic properties, depending on the environment in which they are grown.

This ability to manipulate neuronal phenotype is being used in an attempt to identify cell surface macromolecules that are important in the development or function of adrenergic and cholinergic properties. We have produced monoclonal antibodies against the surface membranes of these neurones and are in the process of characterizing them. Results are presented on the binding specificity of one of these antibodies and on the effect of two other antibodies on neurotransmitter synthesis, uptake, and release.

INTRODUCTION

The precise structural organization of the adult nervous system and its proper functioning depend upon the ability of neurones to make highly ordered synaptic connexions. The molecular mechanisms which regulate the development of these specific connexions are not well understood. However, macromolecules on the cell surface are thought to play an important role in the formation and function of neuronal synapses (Barondes, 1976).

Several approaches have been used to isolate and characterize neuronal surface molecules. Proteins from synaptic membranes obtained by subcellular fractionation of central nervous system tissue have been analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. These studies have identified a variety of proteins from synaptosomes, synaptic junctional complexes and postsynaptic densities (Blomberg, Cohen & Siekevitz, 1977; Cohen *et al.* 1977; Kelly & Cotman, 1977; Yen *et al.* 1977). It has not yet been possible to associate the identified proteins with specific cell types or to discover what role they play in synaptic function.

A second approach has been to use lectins to identify surface glycoproteins and

glycolipids, and differences in lectin binding to specific cell types in various regions of the nervous system have been found (Kelly *et al.* 1976; Pfenninger & Maylie-Pfenninger, 1978). During development cells also exhibit changes in lectin binding (Sieber-Blum & Cohen, 1978; Denis-Donini, Estenoz & Augusti-Tocco, 1978; Hatten, Schachner & Sidman, 1979; Schwab & Landis, 1980). Some lectins bind preferentially to specific parts of the neurone in cultures of chick dorsal root ganglion cells, mouse cerebellum, and rat sympathetic neurones (Denis-Donini *et al.* 1978; Hatten *et al.* 1979; Schwab & Landis, 1980). However, since lectins are not monospecific and bind to a number of different macromolecules, they may be of limited use for the investigation of a molecule's functional role.

The use of immunological techniques to produce antisera against nervous system antigens may provide more specific reagents to define cell surface molecules. Xenogenic immunizations followed by absorption with non-neural tissues and immunizations with neural cell lines, plasma membranes, synaptosomal fractions and purified proteins have produced antisera capable of distinguishing brain regions, neural cell lines, and specific parts of a cell (Akeson & Herschman, 1974; Schachner *et al.* 1975; Stallcup & Cohn, 1976; Lee, Shelanski & Greene, 1977; Woodhams *et al.* 1979; Brookes, Fields & Raff, 1979; Ben-Shaul, Hausman & Moscona, 1979; Sanes *et al.* 1979). More recently, antibody production has been greatly facilitated by a technique introduced by Milstein and colleagues (Köhler & Milstein, 1975; Galfre *et al.* 1977). Myeloma cells are fused with spleen cells from mice immunized with nervous system tissue to produce hybrid cells (see Eisenbarth, Walsh & Nirenberg, 1979; Barnstable, 1980). Each hybridoma clone secretes a continuous supply of antibody specific for a unique antigenic determinant. In principle, the availability of large quantities of monospecific antibodies should allow detailed analyses of the localization, ontogeny, biochemistry, and function of neuronal surface molecules.

Unlimited quantities of highly specific reagents are necessary but not sufficient for precise analyses of the role of cell surface molecules in neuronal recognition and function. Nervous tissue *in vivo* consists of heterogeneous populations of neurones intermingled with glial elements. It is difficult to draw firm conclusions from effects of infusing antibody into this complex system. For example, if an antibody blocks synaptic transmission *in vivo*, it will be difficult to determine whether this effect is caused by binding to a molecule necessary for synaptic function or results from impaired neuronal health which may make neurones leaky (see Williams, Barna & Schupf, 1980). On the other hand, use of homogeneous populations of neurones capable of making synaptic connexions in culture would avoid this ambiguity; it is possible to distinguish antibodies that inhibit synaptic function from those that cause impairment of general neuronal health. For example, after chronic or acute exposure to antibody, assays for synaptic function such as neuronal ability to synthesize, take up and release transmitter, generate and conduct action potentials, and make synapses can be compared to assays for general health such as measurements of neuronal protein, lipid, and resting potentials.

We use primary cultures of dissociated rat sympathetic neurones grown in the virtual absence of non-neuronal cells. These neurones develop adrenergic or cholinergic properties, depending on the environment in which they are grown

(for reviews, see Patterson, 1978, Bunge, Johnson & Ross, 1978; Potter, Landis & Furshpan, this volume). This ability to manipulate neuronal phenotype is being used to identify cell surface molecules that are important in the development or function of adrenergic and cholinergic neurones. We have produced monoclonal antibodies against surface membranes of these neurones. Preliminary results are presented on the binding specificity of one of these antibodies and on the effect of two other antibodies on transmitter synthesis, uptake, and release.

METHODS

Cell preparation

Neuronal cultures were prepared by previously described methods (Mains & Patterson, 1973; Patterson & Chun, 1977*a*). Briefly, sympathetic neurones were dissociated from neonatal rat superior cervical ganglia (SCG), plated on collagen-coated dishes in L-15 CO₂ medium with adult rat serum and nerve growth factor, and treated with 10⁻⁵ M-cytosine arabinoside to kill dividing cells. The neurones were grown in medium containing 20 mM-K⁺ to obtain adrenergic cultures (Walicke, Campenot & Patterson, 1977) or on a monolayer of heart cells or in medium conditioned by them to obtain cholinergic cultures (Patterson & Chun, 1977*a*; Fukada, 1980).

To obtain cultures of ganglionic non-neuronal cells, dissociated SCG cells were cultured in the absence of nerve growth factor and cytosine arabinoside (Patterson & Chun, 1974).

Production of hybridomas and monoclonal antibodies

Four-week-old adrenergic cultures were washed extensively with phosphate buffered saline and emulsified in complete Freund's adjuvant. BALB/c, A/J, or C57BL/6 mice were injected intraperitoneally and subcutaneously with 15–20 cultures containing 2–3 × 10³ neurones each. This was followed 6 to 8 weeks later by a booster intraperitoneal injection without adjuvant. The spleens were removed 3 days later and mechanically dissociated. Spleen cells (10⁸) were fused with P3-NS1/1-Ag4-1 mouse myeloma cells (10⁷) in 40% polyethylene glycol after Galfre *et al.* (1977). The cells were suspended in hypoxanthine/aminopterin/thymidine selective medium (Littlefield, 1964) and distributed at a density of 10⁴ to 10⁵ myeloma cells per well on feeder layers of thymocytes and spleen cells in Falcon multiwell plates. Initially, and after each cloning, hybrid supernatants were assayed for ability to bind to neuronal surfaces by indirect radioimmunoassay (see below). Such antibody-producing hybrids were cloned by limiting dilution (0.3 cell/well) in multiwell plates on feeder layers. The cloned hybrids were grown in flasks or as ascities tumours in mice primed 1 week earlier with 1 ml pristane (2,6,10,14-tetramethylpentadecane). Hybrid supernatants and ascities fluids were eluted from a protein A-Sepharose affinity column (Hjelm, Hjelm & Sjoquist, 1972) or DEAE cellulose after ammonium sulphate precipitation (Hudson & Hay, 1976), concentrated to 1 mg/ml, and stored at -70 °C.

Radioimmunoassay for cell surface antibody binding

The ability of the monoclonal antibodies to bind to neuronal surface membrane was screened by an indirect binding assay performed on ice. Three- to four-week-old neuronal cultures were washed 5–7 times with phosphate buffered saline containing 0.1% bovine serum albumin and incubated for 30–45 min with hybridoma supernatants or ascitic fluid. After washing, the cultures were incubated with ^{125}I -labelled affinity purified rabbit anti-mouse F(ab')_2 for 1–2 h. The cultures were washed, scraped, and counted for radioactivity. Non-specific binding, assessed with supernatants from the parent myeloma or with fresh growth medium, was 100–500 cpm; binding greater than three-fold above background was taken to be positive.

Fluorescence microscopy

Indirect immunofluorescence was used to visualize antibody binding on neurons and other cultured cells. The assay was performed as described above except that the second antibody was rhodamine labelled goat F(ab')_2 fragment anti-mouse IgG (dilution 1:10; Cappel Labs). Rhodamine-specific fluorescence was examined with epi-illumination on a Zeiss 1M 35 inverted microscope equipped with HBO50 mercury burner, a 580 reflector and 510–560 excitation filter. Photographs were taken on Ilford HP5 film with standardized exposure times and processed under identical conditions so that direct comparisons can be made.

Antibody adsorptions

Cell suspensions for absorption were prepared from adult rat tissues immediately before use. Briefly, liver, kidney, spleen, brain, skeletal muscle, or heart tissue was removed and minced. The tissue pieces were then mashed between frosted glass slides. The resulting cell suspensions were filtered through nylon gauze and washed extensively. 200 μl of a 1:2 dilution of N10 supernatant was added to 200 μl of packed cells, incubated for 30 min on ice with mixing every 10 min followed by centrifugation at 1500 rpm for 10 min. Binding of the supernatants to 4-week-old adrenergic neurones was quantitated by radioimmunoassay.

Functional assays

Neurones were grown in L-15 CO_2 medium for 1 week and then in the chronic presence of antibody (10 $\mu\text{g}/\text{ml}$) under adrenergic or cholinergic conditions for an additional 2–3 weeks. The ability of adrenergic neurones to take up and release [^3H]norepinephrine ([^3H]NE) was assayed as described previously (Patterson, Reichardt & Chun, 1976). To measure transmitter uptake, cultures were incubated for 15 or 60 min in 50 nM-1-[^3H]NE, rinsed and counted. To measure transmitter release, cultures were incubated for 60 min in 50 nM-1-[^3H]NE. After washing, they were incubated further in a balanced salt solution (BSS) containing 5 mM-KCl, 3 mM- CaCl_2 , 140 mM-NaCl, 15 mM-HEPES-Tris (pH 7.2), to measure spontaneous release or in the same solution but containing 50 mM-KCl (with NaCl lowered correspondingly) to measure evoked release. Effluent radioactivity was collected periodically and the results expressed as half-time for release from neurones.

The ability of adrenergic and cholinergic cultures to synthesize and accumulate neurotransmitter was determined by incorporation of radioactive precursors, [^3H]tyrosine and [^3H]choline during a 2 h incubation at 37 °C (Mains & Patterson, 1973). The radioactivity in NE, dopamine and acetylcholine was determined after high voltage electrophoresis of the culture extracts.

RESULTS

Antibody N10

Antibody N10 was obtained by the fusion of P3-NS1/1-Ag4-1 (NS1) myeloma cells with spleen cells from a BALB/c mouse immunized with adrenergic sympathetic neurones. N10 hybrid supernatants bind cultures of adrenergic neurones at least seven-fold above background as judged by radioimmunoassay. The hybridoma was cloned once by limiting dilution and all subclones were positive.

Binding specificity of N10 to cultured cells

N10 binds to both cell somas and processes of adrenergic neurones as shown in Fig. 1. Process staining is apparently more uniform (Fig. 1*b*), and cell soma staining seems more intense than process staining (Figs. 1*b* and 1*d*). Increased staining of cell somas as compared to processes is probably not due to a thicker optical section: (1) An optical setting permitting the sharpest image of the cell soma perimeter shows that N10 staining is uneven or 'patchy' (Fig. 1*d*). (2) In cholinergic cultures, N10 does not stain the cell soma more than processes (Fig. 2*b*). Quantitative electron microscopic studies are in progress to directly test this and to localize N10 binding at the ultrastructural level.

Cholinergic sympathetic neurones grown in medium conditioned by heart cells also bind N10 (Fig. 2). Binding to processes appears uniform as with adrenergic neurones (Figs. 2*b* and 2*d*). However, N10 stains cholinergic cell somas less than adrenergic ones; in fact, it is often difficult to distinguish cell soma staining from process staining as shown in Fig. 2*b*. In contrast to adrenergic cultures, where all the cell somas stain with intense fluorescence, there is a marked heterogeneity in cell soma staining for cholinergic cultures. Some cell somas barely stain at all (Fig. 2*b*), while others exhibit graded levels of fluorescence (Fig. 2*d*). However, in no case do any of the cell bodies in cholinergic cultures stain brightly.

A mixed culture of neurones on a dense layer of heart cells is shown in Fig. 3*a*. Under these conditions, neurones synthesize predominantly acetylcholine and make cholinergic synapses with each other (Patterson & Chun, 1977*a*; MacLeish, 1976). In these mixed cultures N10 also binds to the neuronal cell somas and processes. However, as with cholinergic neurone-alone cultures grown in medium conditioned by heart cells, these neuronal somas show marked heterogeneity in N10 staining and always stain less intensely than adrenergic somas. Note that the heart cells are not stained by this antibody, nor are cultured rat fibroblasts (data not shown).

Ganglionic non-neuronal cells from the SCG are shown in Fig. 4*a*, and several different morphologies are present. Some cells are flat and others long and thin, extending processes which resemble neurites. However, the antibody does not stain

any of these cells or their processes (Fig. 4*b*). Similarly, N10 does not stain cultured C6 rat glioma cells (data not shown).

It should be noted that the growth medium containing rat serum used to culture neurones is also used to culture the various non-neuronal cells (heart cells, fibroblasts, ganglionic non-neuronal cells, and C6 glioma). This suggests that N10 is not simply binding to a serum component which binds non-specifically to cells. In addition, collagen-coated dishes incubated at 37 °C for 3–4 weeks with L-15 CO₂ medium containing 20 mM-K⁺ or medium conditioned by heart cells do not bind detectable N10 in radioimmunoassay.

Binding specificity of N10 to adult rat tissue by absorption

The specificity of N10 was further studied by absorption to tissues from adult rats. The antibody was absorbed with an equal volume of packed cells from liver, kidney, spleen, brain, skeletal muscle or heart. After absorption the supernatants were assayed for ability to bind adrenergic cultures by radioimmunoassay. The results show that only brain tissue can successfully absorb out N10 binding activity (Fig. 5).

Antibodies N12 and N14

Spleen cells from an A/J or C57Bl/6 mouse immunized with adrenergic sympathetic neurones were fused with NS1 myeloma cells to generate N12 and N14 respectively. Each hybridoma was cloned once by limiting dilution and all subclones were positive. N12 and N14 bind to somas and processes of neurones grown under both adrenergic and cholinergic conditions as judged by immunofluorescence (data not shown). When neurones are grown in the chronic presence of either N12 or N14, neuronal survival (measured by counting cell somas) is not significantly affected, and the cells retain the ability to generate and conduct action potentials (data not shown).

Effect of N12 and N14 on NE uptake

The ability of adrenergic neurones to take up NE after chronic exposure to antibody was studied by incubating the cultures in low concentrations of ³H-NE. The uptake of ³H-NE into cells increases linearly with incubation time and reaches saturation by 60 min (data not shown). Thus, at 15 min the amount of radioactivity in the cells represents a rate of NE uptake, whereas at 60 min, it represents the extent of NE uptake. Neither the rate nor extent of NE uptake is affected by growth of the neurones in N12 or N14 (Table 1). These data indicate that the number of uptake and storage sites are not affected significantly by the antibodies. In addition, since uptake may be an indirect measure of the amount of axonal membrane present (Iversen, 1967), these results suggest that the antibodies do not affect the ability of neurons to elaborate processes capable of NE uptake.

Effect of N12 and N14 on NE release

The ability of adrenergic neurones to release NE, both spontaneously and upon stimulation by 50 mM-K⁺ was studied to determine whether the antibodies induce membrane leakiness, affect voltage sensitive Ca²⁺ channels or other molecules involved in transmitter release. Cultures were preincubated with ³H-NE and effluent

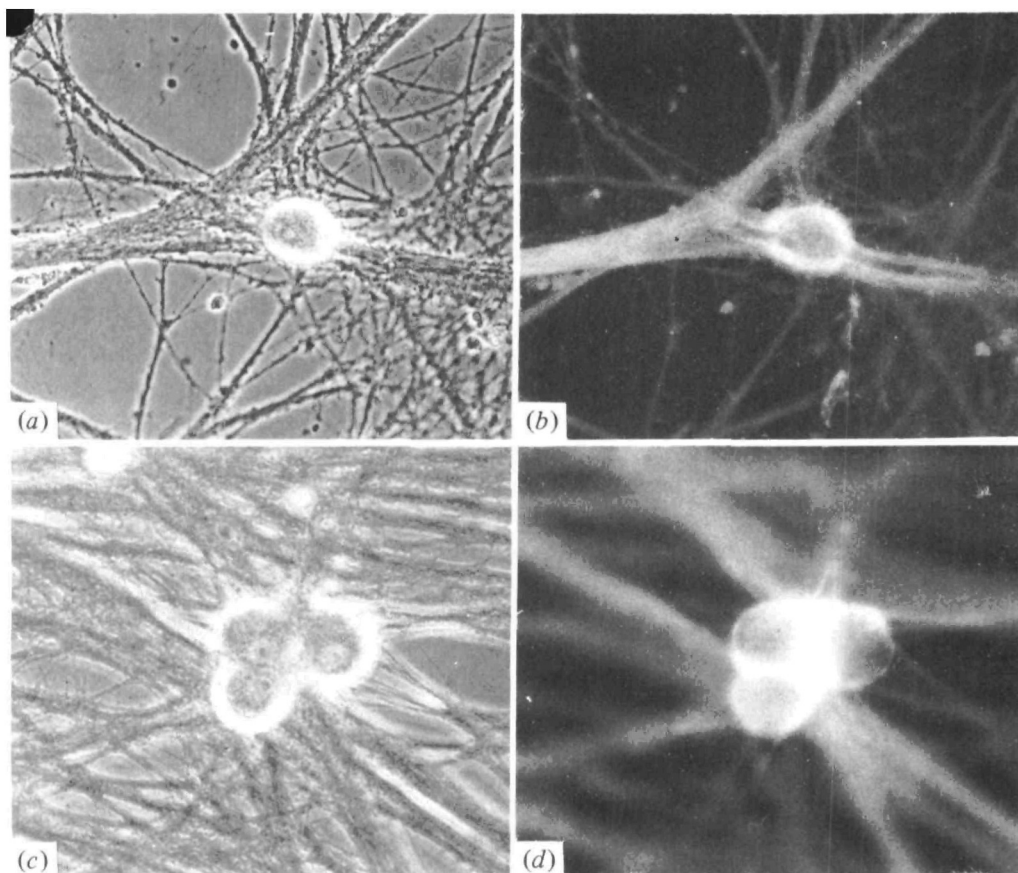


Fig. 1. Immunofluorescent staining of adrenergic sympathetic neurones by N10. (a), (c) Phase contrast micrographs of a 5-week-old adrenergic culture grown in L-15 CO_2 medium containing 20 mM- K^+ . (b) Same field as (a) showing fluorescent staining of cell somas and processes. The small fluorescent dots are cell debris. (d) Same field as (c) showing uneven staining of cell bodies. Focus adjusted to obtain the sharpest image of the cell somas.

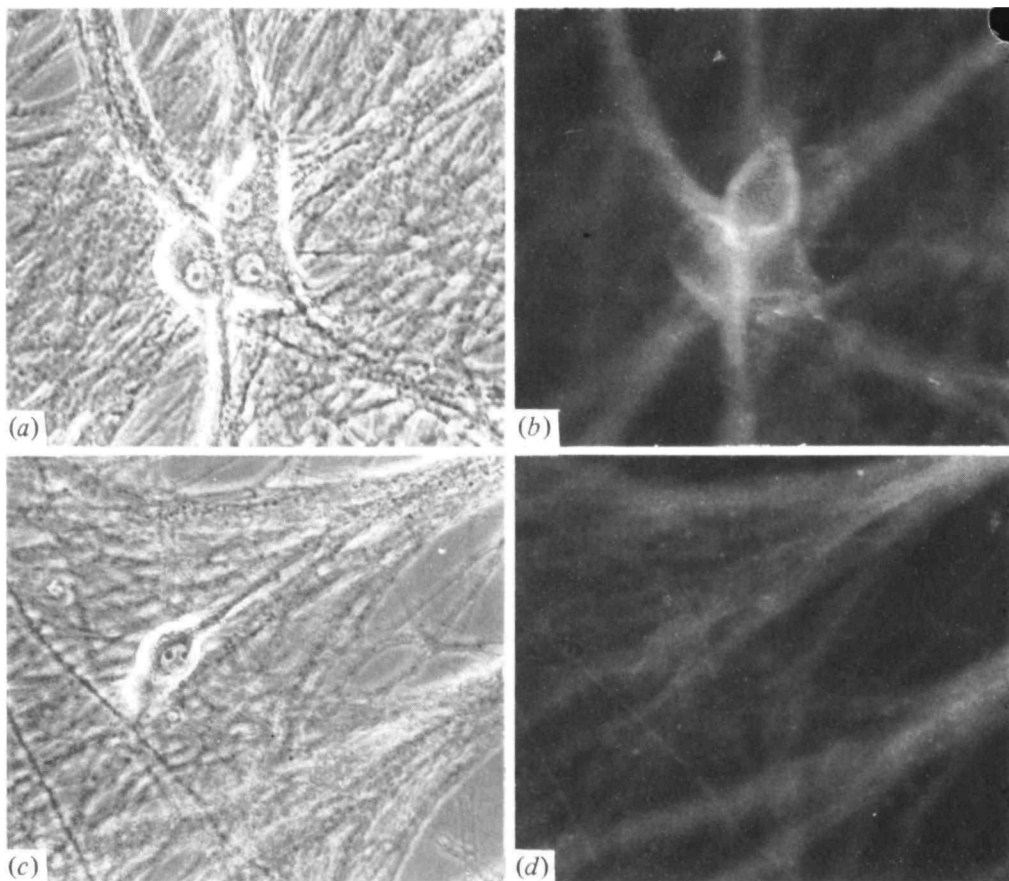


Fig. 2. Immunofluorescent staining of cholinergic sympathetic neurones by Nro. (a), (c) Phase contrast micrographs of a 5-week-old cholinergic culture grown in medium conditioned by heart cells (sister to the culture in Fig. 1). (b) Same field as (a) showing barely distinguishable cell soma staining. (d) Same field as (c) showing fluorescent processes and variable uneven, less intense staining of cell somas.

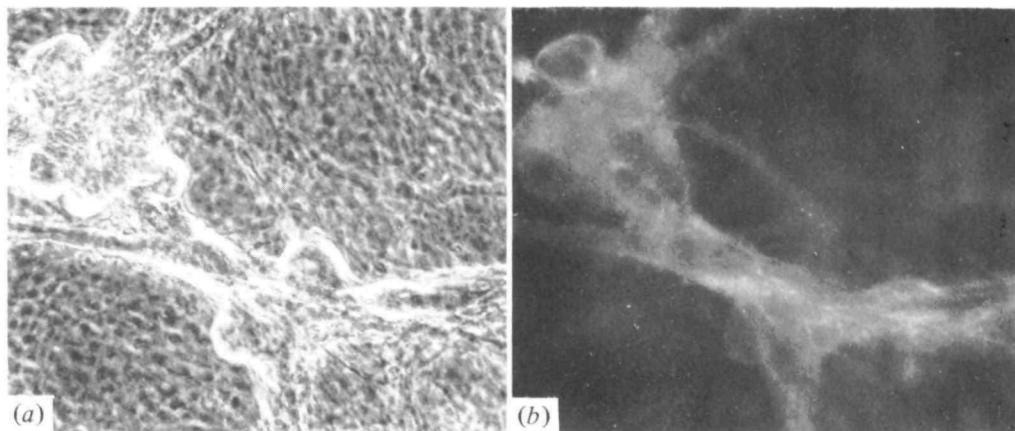


Fig. 3. Immunofluorescent staining of a 4-week-old mixed neurone-heart cell culture by Nro. (a) Phase-contrast micrograph. (b) Same field showing faint and heterogeneous fluorescent staining of the heart cells.

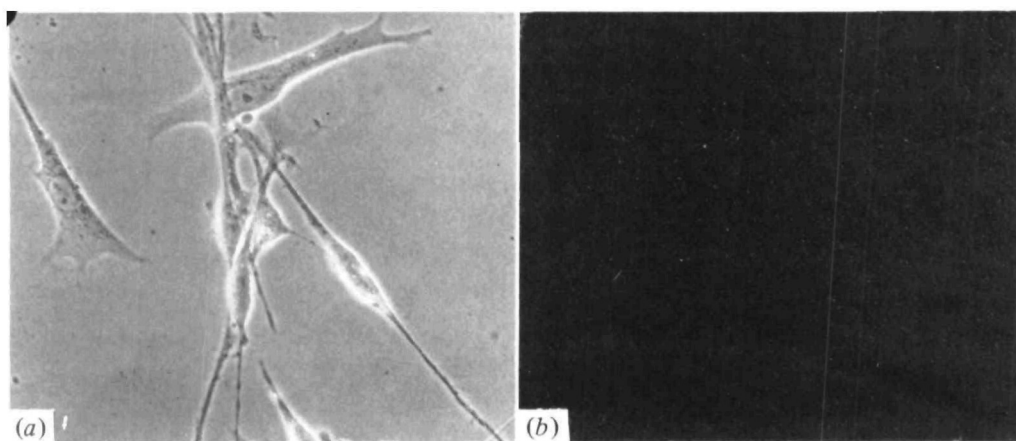


Fig. 4. Immunofluorescent staining of a 4-week-old ganglionic non-neuronal cell culture by N10. (a) Phase-contrast micrograph. (b) Same field showing no staining of non-neuronal cells from the SCC even though some of the cells elaborate processes which superficially resemble neurites.

Table 1. *Effect of antibodies on NE uptake*

	15 min (rate)*	60 min (extent)*
Control	34370	108460 ± 3570
N14	33820	118250 ± 11030
N12	35820	111400 ± 5400

* cpm/culture. Cultures were grown for 7 days in L-15 CO₂ medium and then with or without antibody (10 µg/ml) for an additional 16 days.

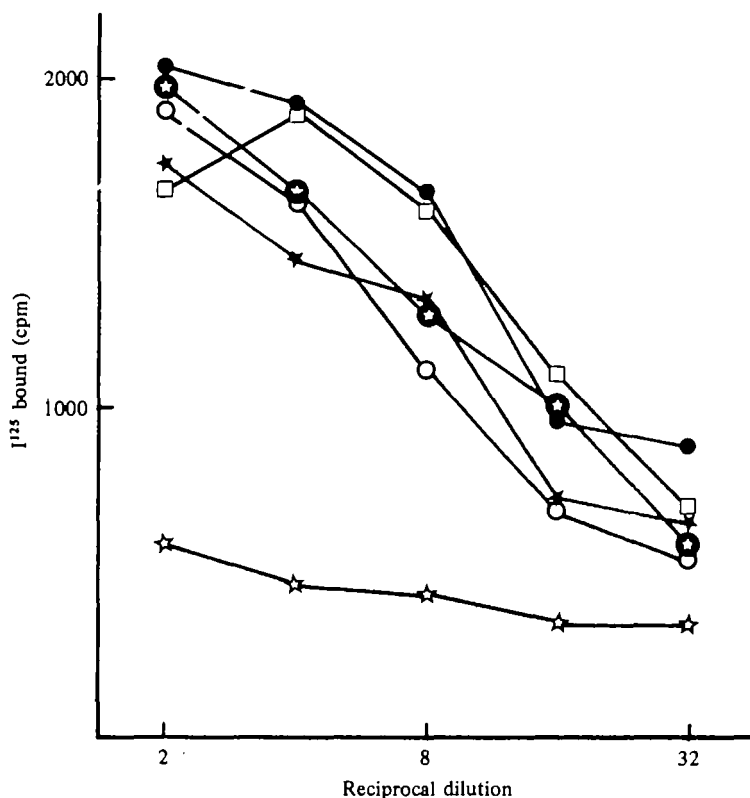


Fig. 5. Residual binding of N10 to adrenergic neurones after absorption with cells from adult rat tissues: ●, liver; ★, kidney; ●, heart; □, spleen; ○, skeletal muscles; ★, brain. A background of 500 cpm was subtracted from all points.

radioactivity monitored in 5 mM-K⁺ BSS to measure spontaneous release, or in 50 mM-K⁺ BSS to measure evoked release. Previous studies have shown that spontaneous release is not Ca²⁺-dependent and that radioactivity is primarily in deaminated products. On the other hand, evoked release is Ca²⁺-dependent, blocked by Co²⁺ and high Mg²⁺, and the radioactivity is primarily in unaltered NE (Patterson *et al.* 1976). As shown in Table 2, neither N12 nor N14 have an effect on the half-times of either spontaneous or evoked release.

Table 2. *Effect of antibodies on NE release*

	Spontaneous*	Evoked*
Control	192	60 ± 3
N14	213 ± 26	61 ± 4
N12	193 ± 5	61 ± 1

* Half-times. Cultures were grown for 7 days in L-15 CO₂ medium and then with or without antibody (10 µg/ml) for an additional 16 days.

Table 3. *Effect of antibodies on transmitter synthesis and accumulation*

A. L-15 CO ₂	ACh*	CA*	ACh/CA
Control	6.83 ± 0.86	9.40 ± 0.80	0.71 ± 0.04
N14	6.26 ± 0.28	7.40 ± 0.66	0.79 ± 0.06
N12	3.66 ± 0.62	5.59 ± 0.54	0.65 ± 0.08
B. CM			
Control	17.73 ± 1.97	2.31 ± 0.25	7.66 ± 0.29
N14	19.56 ± 2.90	2.87 ± 0.53	6.61 ± 0.48
N12	8.51 ± 0.67	1.59 ± 0.08	5.32 ± 0.19

* pmol/culture. Cultures were grown for 7 days in L-15 CO₂ medium and then with or without antibody (10 µg/ml) for an additional 20 days, with or without CM.

Effect of N12 and N14 on transmitter synthesis and accumulation

The ability of adrenergic and cholinergic cultures to synthesize and accumulate neurotransmitter was studied by incubating the cultures with the radioactive precursors, ³H-tyrosine and ³H-choline, and analysing the culture extract for ³H-catecholamine (CA; ³H-NE and ³H-dopamine) and ³H-acetylcholine (³H-ACh). This assay measures the net total of a variety of transmitter functions such as uptake of precursors, levels of cofactors, levels of synthetic enzymes, ability to store transmitter and to degrade it (Mains & Patterson, 1973; Patterson & Chun, 1977a). The results of this experiment are shown in Table 3.

In part A, neurones were grown in L-15 CO₂ medium which produces primarily adrenergic cultures although it is not the most adrenergic condition that can be achieved. In fact, the cultures also synthesize considerable ACh as well. The ratio of ACh to CA is a measure of the transmitter character of the culture (see Patterson & Chun, 1977a). Cultures grown chronically in N14 are not significantly affected by the antibody. They produce near control levels of both transmitters. However, cultures grown in N12 show a 50% decrease in ACh production and a 50% decrease in CA production. This decrease affected both transmitters to approximately the same extent since the ACh/CA was not significantly altered.

Cultures grown in medium conditioned by heart cells are primarily cholinergic but synthesize low levels of CA (Patterson & Chun, 1977a). As shown in part B, N14 does not affect transmitter production significantly. However, N12 again causes decreases in both ACh (50%) and CA (30%) production when compared to control values.

DISCUSSION

Antibody N10

These results show that N10 binds uniformly to both adrenergic and cholinergic neurites. It is difficult with immunofluorescence techniques to determine if there is a quantitative difference in binding to processes in the two type of cultures. However, there is an obvious difference in staining of somas; N10 stains adrenergic cell bodies more intensely than cholinergic ones. As these neurones develop cholinergic properties, they gradually lose adrenergic properties (Patterson & Chun, 1977*b*; Landis, 1980). This difference in binding may reflect the gradual loss of the antigen as the neurones become cholinergic. In addition, the heterogeneity in cholinergic soma staining may reflect neurones in different stages of conversion from adrenergic to cholinergic phenotype. This heterogeneity is being investigated with single cell microcultures (see Potter *et al.* this volume) using combined electrophysiological and immunofluorescent techniques. There is also an uneven distribution of N10 staining on adrenergic cell bodies. This may be due to an uneven distribution of molecules which N10 recognizes on adrenergic somas. Another possibility is that it results from a high concentration of localized adrenergic endings on the cell soma and possibly dendrites. This would be consistent with serial section reconstruction studies by Landis (1977) which show that for these neurones, synapses end either on cell somas or dendrites but not on axons. We are currently examining these possibilities at the ultrastructural level.

Of the various tissues tested, only brain was able to absorb out the antibody binding activity. Studies of binding to frozen sections are currently underway to determine which cells in the central nervous system bind this antibody. These absorption studies demonstrate that N10 does not bind to determinants expressed on all rat tissues. The possibility that N10 is neurone-specific is supported by the fact that the antibody does not bind ganglionic non-neuronal cells from the SCG, cultured heart cells, fibroblasts, or C6 glioma cells. This possibility is being tested using histological techniques.

Antibodies N12 and N14

These preliminary functional studies illustrate the approach we are taking to identify cell surface molecules important in neuronal function. They show that: (1) Not every antibody which binds the neurones will cause a specific decrease in transmitter production. A number of antibodies do not, and N14 is an example of one of these. (2) This effect of N12 on transmitter production is a relatively specific one because neuronal survival and growth are not inhibited. Furthermore, N12 does not affect NE uptake or release by adrenergic cultures but does decrease their ability to produce both CA and ACh. Although we do not yet know where the site of action is, it probably does not involve cofactors necessary for transmitter synthesis. These requirements are different for adrenergic and cholinergic biosynthetic pathways, and some mechanism common to both pathways must be involved. One possibility is that N12 affects Na-dependent precursor uptake. If the availability of ³H-tyrosine or ³H-choline were limiting, transmitter synthesis would be decreased. Another

possibility is that N12 decreases the rate of spontaneous neuronal activity in these cultures, which results in decreased activity of transmitter biosynthetic enzymes (Weiner & Rabadjija, 1968; Black, Hendry & Iversen, 1971). We are currently doing experiments to test these alternatives.

This preliminary report shows that it is possible to produce monoclonal antibodies against cultured sympathetic neurones. We are primarily interested in the ability of these antibodies to block a variety of neuronal functions. Studies are underway to determine whether acute or chronic exposure of neurones to these antibodies will affect the generation and conduction of action potentials, synaptic transmission, neurotransmitter choice and other functions.

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